

Epicardial FSTL1 Reconstitution Regenerates the Adult Mammalian Heart

Ke Wei^{1*}, Vahid Serpooshan^{2*}, Cecilia Hurtado¹, Marta Diez-Cuñado^{1,2}, Mingming Zhao², Sonomi Maruyama³, Wenhong Zhu¹, Giovanni Fajardo², Michela Nosedà⁴, Kazuto Nakamura³, Xueying Tian⁵, Qiaozhen Liu⁵, Andrew Wang², Yuka Matsuura², Paul Bushway¹, Wenqing Cai¹, Alex Savchenko¹, Morteza Mahmoudi^{2,6}, Michael D. Schneider⁴, Maurice vanden Hoff⁷, Manish J. Butte², Phillip C. Yang², Kenneth Walsh³, Bin Zhou^{5,8}, Daniel Bernstein², Mark Mercola¹, and Pilar Ruiz-Lozano²

*Authors contributed equally

¹ Department of Bioengineering, University of California, San Diego, La Jolla, CA 92037 and Sanford-Burnham-Prebys Medical Discovery Institute, 10901 N. Torrey Pines Road, La Jolla, CA 92037

² Stanford Cardiovascular Institute and Department of Pediatrics, Stanford University School of Medicine, 300 Pasteur Dr., Stanford, CA 94305

³ Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, MA 02118

⁴ Imperial College London, Faculty of Medicine, Imperial Centre for Translational and Experimental Medicine, Du Cane Road, London W12 0NN

⁵ Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, and Shanghai Institutes for Biological Sciences, Graduate School of the Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai, 200031, China

⁶ Nanotechnology Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

⁷ Academic Medical Center. Dept Anatomy, Embryology and Physiology. Meibergdreef 15. 1105AZ Amsterdam The Netherlands

⁸ CAS Center for Excellence in Brain Science, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

Address correspondence to: Pilar Ruiz-Lozano, Department of Pediatrics, School of Medicine, Stanford University, 300 Pasteur Dr., Stanford 94305; email: prlozano@stanford.edu

Running Title: Engineered Epicardium Activates Cardiac Regeneration

Summary

The elucidation of factors that activate the impaired regeneration of the adult mammalian heart is of major scientific and therapeutic importance. We found that epicardial cells contain a potent cardiogenic activity identified following mass spectrometric analysis as follistatin-like 1 (Fstl1). Epicardial Fstl1 declines following MI and is replaced by myocardial expression. Myocardial Fstl1 does not promote regeneration, either basally or upon transgenic overexpression. Restoration of FSTL1 via an epicardial patch stimulates cell cycle entry and division of pre-existing cardiomyocytes, improving cardiac function and survival in mouse and swine models of myocardial infarction. FSTL1 is the first example of an endogenous epicardial protein that stimulates adult mammalian heart regeneration. The data suggest that the loss of epicardial FSTL1 is a maladaptive response to injury, and that its restoration would be an effective way to reverse myocardial death and remodeling following MI in humans.

The epicardium of the heart is an external epithelial layer that contributes to myocardial growth during development by providing progenitor cells^{1,2} as well as mitogens, including FGFs, IGF2, and PDGFs³⁻⁵. Recent studies suggest that the epicardium might also preserve function of the adult myocardium following injury, possibly as a source of myogenic progenitors^{6,7}. No epicardial-secreted factors have shown to support adult myocardial regeneration in mammals.

Epicardial signal activates cardiomyocyte division

We co-cultured an epicardial mesothelial cell (**EMC**) line with Myh6⁺ mouse embryonic stem cell-derived cardiomyocytes (referred to as **mCMs^{ESC}**; **Extended Data Fig. 1, Suppl. Videos 1, 2, and Methods**). Co-cultures consistently increased the number of cardiomyocytes (α -actinin⁺ cells, **Fig. 1a-c**) and the expression of cardiomyocyte markers (**Fig. 1d**). Conditioned media from EMC cultures recapitulated this effect (**Fig. 1e-h**). The number of α -actinin⁺ cells exhibiting rhythmic Ca²⁺ transients also increased with the addition of EMC media (8.6-fold) (**Fig. 1i**), as quantified automatically by Kinetic Imaging Cytometry, (Vala Sciences). Similarly, conditioned media prepared from adult epicardial-derived cells⁸ (**Fig. 1j**) increased proliferation and nearly doubled the incidence of Aurora B Kinase in the cleavage furrow connecting adjacent embryonic cardiomyocytes (Tnnt2⁺ cells; 0.19 to 0.33%, $P < 0.05$, **Fig. 1k-m**), indicating a secreted activity in the adult epicardium that promotes cytokinesis of embryonic cardiomyocytes.

Engineered epicardium improves cardiac function after myocardial infarction

We next evaluated the effect of epicardial-secreted factors in the adult injured heart by delivering conditioned media in 3D-collagen nano-fibrillar patches⁹. Patches were designed with an elastic modulus emulating the embryonic epicardium ($E \sim 12$ kPa)⁹, lower than the mature epicardium ($E > 30-40$ kPa) and fibrotic cardiac tissue ($E > 100$ kPa), but higher than those for the most currently used scaffolding biomaterials ($E \leq 1$ kPa) (**Fig. 1n, o**). Patches seeded with EMC-media (33 volume %) were sutured onto the heart immediately following surgical-induced myocardial infarction (**MI**, permanent ligation of the Left Anterior Descending LAD coronary artery, **Fig. 1p, q**). Two weeks later, patch-treated hearts (both with or without EMC-media) showed improved morphometric parameters (**Fig. 1r-t** and **Extended Data Table 1**), consistent with collagen patch providing a mechanical support that inhibits remodeling⁹. Notably, only patch-EMC-media treatment improved cardiac function (**Fig. 1r, s** and **Extended Data Table 1**).

Fstl1 is an epicardial factor that induces cardiomyocyte proliferation

To identify bioactive proteins, we analyzed EMC-conditioned media by mass spectrometry. Comparison of spectra to the IPI rat database identified 1596 peptide reads corresponding to 311 unique proteins. Ten secreted proteins with the highest spectral counts were selected for testing in the mCMs^{ESC} assay. Of these, cardiogenic activity was noted only with Follistatin-like-1 (also known as Fstl1, FRP or TSC36, Accession number: NP_077345.1) (**Fig. 2a**). Unlike Follistatin, Fstl1 does not block Activin and its biochemical and biological functions are poorly characterized¹⁰. Fstl1 levels increase in the blood stream following acute MI and, for this reason, it has been considered a biomarker for acute coronary syndrome¹¹.

Treating mCMs^{ESC} for 8 days with bacteria-synthesized recombinant human FSTL1 (10 ng/ml) increased the number of cardiomyocytes (**Fig. 2b-d**), the transcription levels of myocardial-specific proteins (*myh6*, *mhc2v*, and *mhc2a*, **Fig. 2e**), and the number of α -actinin⁺ cells with rhythmic Ca²⁺ transients (**Fig. 2f**). FSTL1 treatment did not induce hypertrophy. Indeed FSTL1 decreased myocyte cell size in a dose-dependent manner (**Fig. 2g**) in 48 hours. Thus, FSTL1 recapitulates the cardiomyogenic activity of the epicardial conditioned medium.

Dynamic expression of Fstl1 after ischemic injury

During fetal development, endogenous Fstl1 is expressed throughout the myocardium of the primitive heart tube¹², but becomes restricted to the epicardium by mid-gestation (**Fig. 2h**). Epicardial expression persists throughout adulthood (**Fig. 2i-k**). Remarkably, Fstl1 localization shifts dramatically following ischemic injury, such that it becomes abundant in the myocardium (**Fig. 2i-l**) and absent in the epicardium and infarcted area (**Fig. 2i,l**).

Epicardial reconstitution of FSTL1 promotes regeneration

Prior studies showed that transient overexpression of Fstl1, either by myocardial transgenesis (Fstl1-TG¹³, **Extended Data Fig. 2a, b**) or systemic infusion, is anti-apoptotic following acute ischemia-reperfusion^{14,15}. In the context of permanent myocardial infarction, myocardial Fstl1-TG mice did not recapitulate the effect of the patch containing EMC-media (**Extended Data Fig. 2a-j**). We next assessed epicardial-hFSTL1 delivery by collagen patches loaded with 10 μ g of recombinant bacterial-synthesized hFSTL1/patch. Patches retained immune-detectable hFSTL1 up to 21 days *in vitro*, and 28 days *in vivo*, the longest times tested (**Extended Data Fig. 3a-f**). Application of hFSTL1-loaded patches simultaneously with MI significantly improved survival (**Fig. 3a**) and sustained long-term recovery of cardiac function (**Fig. 3b** and **Extended Data Table 1**).

Epicardial patch+FSTL1 also improved cardiac function when applied onto infarcted hearts of Fstl1-TG mice (**Fig. 3c**); thus, myocardial overexpression of FSTL1 is insufficient for long-term recovery but epicardial reconstitution of recombinant FSTL1 is necessary to induce the beneficial effects.

The improved cardiac function and survival following patch+FSTL1 treatment was accompanied by attenuated fibrosis (**Fig. 3d-e**, **Extended Data Fig. 3g-j**, **Extended Data Table 1**, and **Suppl. Videos 3-5**), increased vascularization of the patch and underlying myocardium at the border of the infarcted region (**Fig. 3f-i**), as reflected by the increased area occupied by vessels (**Fig. 3f, g**) and the increased number of vessels (of any size) per area unit (**Fig. 3h, i**). Masson's trichrome staining showed contiguous engraftment of the patch+FSTL1 onto the host myocardium and demonstrated migration of host cells into the patch including evidence of striated cells (green arrows, last two columns in **Extended Data Fig. 3k**).

A similar recovery was found when FSTL1-loaded patches were grafted one week after ischemia/reperfusion injury in the mouse, when cardiac function had substantially decreased (about 15% reduction in FS%). As is typical, cardiac function of untreated animals progressively declined (22%, 20% and 16% FS at 1, 3 and 5 weeks post-I/R). In contrast, the patch+FSTL1 cohort showed a nearly complete and stable recovery of FS (to 34% three weeks post-I/R) (**Extended Data Fig. 4a-d** and **Extended Data Table 2**), suggesting that epicardial-delivered FSTL1 is sufficient to revert the loss of cardiac function after experimental MI.

FSTL1 induces cardiomyocyte cell cycle entry *in vivo*

Four weeks following MI, the patch+FSTL1 cohort showed evidence of striated myocytes (α -actinin⁺ cells) within the patch (**Extended Data Fig. 5a-d**). Cardiomyocytes in the border zone had undergone cell division (**Extended Data Fig. 5e-h**) by several independent criteria, including an increased number of double-positive α -actinin⁺, phospho-Histone H3 (pH3)⁺ cells (**Fig. 3j-l**; and **Extended Data Fig. 5e-h, i-k**), increased incidence of Aurora B kinase localized to the midbody between α -actinin⁺ cells (**Fig. 3m-n** and **Suppl. Video 6**), and increased incidence of cells that were double-positive for pH3 and the nuclear cardiomyocyte maker PCM1¹⁶ (**Fig. 3o, p**) relative to MI and patch-only cohorts (**Extended Data Fig. 5l-r**). Thus, epicardial FSTL1 delivery activates cardiomyocyte cell cycle entry and cytokinesis *in vivo* reminiscent of the *in vitro* results above. Proliferating cardiomyocytes were found in the border zone and, to a lesser extent, the infarcted area, but not remotely in the myocardium (**Extended Data Fig. 5s, t**). Increased

cardiomyocyte proliferation was also observed in the I/R injury model with delayed patch implantation (**Extended Data Fig. 4e, f**). Notably, FSTL1 did not diminish cardiomyocyte apoptosis, the extent of the infarcted area or the area at risk (hypoperfused area) acutely after MI; nor did it affect apoptosis or inflammation at day 4 and day 8 post-MI (**Extended Data Fig. 6**).

In contrast to patch+FSTL1 delivery, transgenic overexpression of *Fstl1* (*Fstl1*-TG mice) did not show any evidence of cardiomyocyte proliferation after MI (**Extended Data Fig. 2k,l**), despite increased vascularization described previously¹³ (**Extended Data Fig. 2m,n**), indicating that epicardial-delivered FSTL1 might function differently than myocardial-expressed *Fstl1*.

To distinguish whether the FSTL1-responsive cells arise from pre-existing myocytes (*Myh6*⁺ cells) or *de novo* from a progenitor population, we heritably labeled *Myh6*⁺ cardiomyocytes using a 4-OH-tamoxifen-inducible *Cre*¹⁷ prior to injury (**Fig. 3q**). 4-OH tamoxifen injected into *Myh6*^{m^{ER}Cre^{ER}}:*Rosa26*^{Z/EG} mice¹⁷ efficiently labeled pre-existing cardiomyocytes with eGFP prior to MI (**Fig. 3r**). Four weeks after patch engraftment, eGFP⁺, pH3⁺ double-positive cells were visible in the infarct area and border zone (**Fig. 3s-v**), indicating that the proliferating cardiomyocytes expressed *Myh6* prior to MI. We treated cardiomyocytes at different stages of differentiation with FSTL1 in order to determine which stage(s) can respond by proliferating. Neither adult mouse cardiomyocytes (**Extended Data Fig. 7a-f**), neonatal rat cardiomyocytes (**Extended Data Fig. 7g-j**) nor cardiomyogenic progenitor cells (*Lin*⁻, *Sca1*⁺, *SP*⁺)¹⁸ responded (**Extended Data Fig. 7k-m**). Of the cells tested, only immature cardiomyocytes (mCMs^{ESC}) proliferated in response to FSTL1 (**Fig. 4a-f**).

It remained paradoxical that neither the endogenous *Fstl1* induced by MI nor myocardially overexpressed *Fstl1* could induce a regenerative response (**Fig. 3, Extended Data Fig. 2**). Western blot analysis indicated that myocardially overexpressed *Fstl1* (in neonatal rat ventricular myocytes, NRVC) migrates substantially slower in SDS-polyacrylamide gels than does epicardial-synthesized *Fstl1* (EMC), and that tunicamycin treatment eliminates the difference (**Fig. 4g**), suggesting cell-type specific glycosylation. The bacterially produced recombinant human FSTL1 (as used in the patch) showed a faster migration consistent with less extensive glycosylation (**Fig. 4h, i**). Direct comparison of recombinant human FSTL1 produced in bacterial versus mammalian cells (NS0-derived mouse myeloma cell line) revealed that mammalian-expressed FSTL1, but not bacterial FSTL1, protects mCMs^{ESC} from H₂O₂-induced apoptosis (**Fig. 4j**), consistent with evidence of cardioprotection¹⁴ but not regeneration (**Extended Data Fig. 2a-j**) in *Fstl1* TG mice.

In contrast, bacterially synthesized human FSTL1 promotes mCMs^{ESC} proliferation, whereas human FSTL1 produced in NS0-derived cells or NRVCs cannot stimulate proliferation of mCMs^{ESC} (**Fig. 4k, l, m, n**). Thus, whether FSTL1 induces cardioprotection versus proliferation correlates with cell source and might reflect post-translational modification.

Epicardial FSTL1 in a preclinical swine model.

Epicardial delivery of FSTL1 was evaluated in the swine model of I/R injury. I/R decreased left ventricular EF from ~50% prior to MI, as determined by magnetic resonance imaging (MRI), to ~30% at 1 week after injury. Application of the patch+FSTL1 to the epicardium over the injured tissue at this time stimulated recovery of contractile function (to ~40% EF) in 2 weeks (3 weeks post-MI I/R) (**Fig. 5a, b**). The recovery remained stable for an additional 2 weeks, the longest time analyzed, and was in contrast to the steady decline seen without treatment or following treatment with patch alone (**Fig. 5b**). FSTL1-treated pigs demonstrated the least scar size of all treatments, including the patch-only condition [see representative MRI images (**Fig. 5c, d**)]. Examination of histological sections of tissues 4 weeks after patch implantation confirmed the limited fibrosis and showed integration of the patch into the host tissue (**Fig. 5e**). Cardiomyocytes in the border zone and ischemic area of the Patch+FSTL1 treated hearts also had evident EdU labeling (**Fig. 5i-m**) and midbody-localized Aurora B kinase (indicative of cytokinesis) (**Fig. 5n**). Vascular smooth muscle cells were also EdU⁺ suggestive of arteriogenesis (**Fig. 5g, h**). Thus, the patch+FSTL1 appears therapeutic in the swine MI I/R model.

Discussion

Heart regeneration studies in zebrafish suggested that the epicardium is activated by injury to produce factors and cells that sustain cardiac function¹⁹. Unlike lower vertebrate hearts, which are robustly regenerative, the mammalian heart retains negligible regenerative potency in adulthood and, instead, sustains cardiomyocyte death and scarring following injury. Very little is known of the endogenous mechanisms that limit regeneration and the topic remains a subject of intense therapeutic interest and scientific debate²⁰. Our data suggest a new view of epicardial function after injury in the mammalian heart. Rather than activation to support cardiac function, the loss of epicardial FSTL1 expression after injury, and the functional and anatomical recovery by reconstitution in an engineered biomaterial, indicate that ischemic injury induces a maladaptive loss of FSTL1 in the epicardium.

We sought to identify the cell population that proliferates in response to FSTL1. FSTL1 could not stimulate mature adult ventricular cardiomyocytes to synthesize DNA or divide, nor did it induce hypertrophy (as can occur in response to mitogens) either at 48 hours (**Fig. 2g**) or 4 weeks post-MI (**Ext Fig. 5o**). In contrast, FSTL1 stimulated replication of newly emerging cardiomyocytes from mouse ESC cultures (**Fig. 4a-f**). FSTL1 did not enhance replication of either ESC-derived progenitors prior to the appearance of *Myh6* (not shown) or a population of cardiac progenitors isolated from the adult murine heart (**Extended Data Fig. 7k-m**), suggesting that competence to respond to FSTL1 occurs transiently. Although at least some mononuclear adult cardiomyocytes can be induced to divide²¹, the FSTL1-responsive cardiomyocytes in our experiments have even less mature sarcomeric and electrophysiological properties²² (e.g. automaticity, relatively high maximum diastolic potential, and slow action potential peak V_{max}) (**Extended Data Fig. 1**). The cells that respond to FSTL1 might overlap cells identified in an earlier analysis of infarcted hearts labeled with *Myh6-Cre*, in which a minor population of *Cre*-labeled cells were reported to divide and give rise new cardiomyocytes upon ischemic injury²³. Whether the FSTL1-responsive cells reflect resident *Myh6+* recruited upon injury e.g.²³ or derive from de-differentiation²⁴ (thus recapitulating the Zebrafish model²⁵) is an interesting question whose resolution will depend on improved method to identify and/or isolate such cells.

Myocardial *Fstl1* induced by MI cannot promote a regenerative response, either basally or when abundantly overexpressed transgenically in cardiomyocytes (**Extended Data Fig. 2**). However, transgenic myocardial *Fstl1* is cardioprotective post-MI¹⁴. Direct comparison of *Fstl1* overexpressed in cardiomyocytes versus the epicardial (EMC) protein revealed tunicamycin-sensitive differences in SDS-PAGE mobility (**Fig. 4**), consistent with the possibility of differential glycosylation (or other post-translational modification) depending on the cell in which it is expressed. We infer from these data that native epicardial and myocardial FSTL1 have analogous differences in glycan structure that affect their function. It will be important to determine the structure of the glycans, as well as elucidate how post-translation modifications dictate whether FSTL1 promotes anti-apoptosis (myocardial) or cardiomyocyte proliferation (EMC and bacterially produced).

These studies identified FSTL1 as a regenerative factor that is normally present in healthy epicardium, but lost upon MI suggesting a mechanism whereby injury maladaptively diminishes the regenerative potency of the mammalian heart. Reconstitution of FSTL1 by an engineered

epicardial biomaterial improved cardiac function in the mouse MI, mouse MI I/R and preclinical swine MI I/R models with evidence of cardiomyocyte regeneration amenable to clinical translation.

References

- 1 van Wijk, B., Gunst, Q. D., Moorman, A. F. & van den Hoff, M. J. Cardiac regeneration from activated epicardium. *PLoS One* **7**, e44692, doi:10.1371/journal.pone.0044692 (2012).
- 2 Cai, C. L. *et al.* A myocardial lineage derives from Tbx18 epicardial cells. *Nature* **454**, 104-108, doi:10.1038/nature06969 (2008).
- 3 Lavine, K. J. & Ornitz, D. M. Rebuilding the coronary vasculature: hedgehog as a new candidate for pharmacologic revascularization. *Trends in cardiovascular medicine* **17**, 77-83, doi:10.1016/j.tcm.2007.01.002 (2007).
- 4 Brade, T. *et al.* Retinoic acid stimulates myocardial expansion by induction of hepatic erythropoietin which activates epicardial Igf2. *Development* **138**, 139-148, doi:10.1242/dev.054239 [pii] 10.1242/dev.054239 (2011).
- 5 Mellgren, A. M. *et al.* Platelet-derived growth factor receptor beta signaling is required for efficient epicardial cell migration and development of two distinct coronary vascular smooth muscle cell populations. *Circ Res* **103**, 1393-1401, doi:10.1161/CIRCRESAHA.108.176768 (2008).
- 6 Smart, N. *et al.* Myocardial regeneration: expanding the repertoire of thymosin beta4 in the ischemic heart. *Ann N Y Acad Sci* **1269**, 92-101, doi:10.1111/j.1749-6632.2012.06708.x (2012).
- 7 Kikuchi, K. *et al.* tcf21+ epicardial cells adopt non-myocardial fates during zebrafish heart development and regeneration. *Development* **138**, 2895-2902, doi:10.1242/dev.067041 (2011).
- 8 Zhou, B. *et al.* Adult mouse epicardium modulates myocardial injury by secreting paracrine factors. *J Clin Invest* **121**, 1894-1904, doi:10.1172/JCI45529 (2011).
- 9 Serpooshan, V. *et al.* The effect of bioengineered acellular collagen patch on cardiac remodeling and ventricular function post myocardial infarction. *Biomaterials* **34**, 9048-9055, doi:10.1016/j.biomaterials.2013.08.017 (2013).
- 10 Tanaka, M. *et al.* DIP2 disco-interacting protein 2 homolog A (Drosophila) is a candidate receptor for follistatin-related protein/follistatin-like 1--analysis of their binding with TGF-beta superfamily proteins. *The FEBS journal* **277**, 4278-4289, doi:10.1111/j.1742-4658.2010.07816.x (2010).
- 11 Widera, C. *et al.* Identification of Follistatin-Like 1 by Expression Cloning as an Activator of the Growth Differentiation Factor 15 Gene and a Prognostic Biomarker in Acute Coronary Syndrome. *Clinical chemistry*, doi:10.1373/clinchem.2012.182816 (2012).
- 12 Adams, D., Larman, B. & Oxburgh, L. Developmental expression of mouse Follistatin-like 1 (Fstl1): Dynamic regulation during organogenesis of the kidney and lung. *Gene Expression Patterns* **7**, 491-500 (2007).
- 13 Shimano, M. *et al.* Cardiac myocyte follistatin-like 1 functions to attenuate hypertrophy following pressure overload. *Proceedings of the National Academy of Sciences of the United States of America* **108**, E899-906, doi:10.1073/pnas.1108559108 (2011).
- 14 Oshima, Y. *et al.* Follistatin-Like 1 Is an Akt-Regulated Cardioprotective Factor That Is Secreted by the Heart. *Circulation* **117**, 3099-3108, doi:10.1161/circulationaha.108.767673 (2008).

- 15 Ogura, Y. *et al.* Therapeutic impact of follistatin-like 1 on myocardial ischemic injury in preclinical models. *Circulation* **126**, 1728-1738, doi:10.1161/CIRCULATIONAHA.112.115089 (2012).
- 16 Bergmann, O. *et al.* Identification of cardiomyocyte nuclei and assessment of ploidy for the analysis of cell turnover. *Experimental Cell Research* **317**, 188-194, doi:<http://dx.doi.org/10.1016/j.yexcr.2010.08.017> (2011).
- 17 Sohal, D. S. *et al.* Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res* **89**, 20-25 (2001).
- 18 Oh, H. *et al.* Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12313-12318 (2003).
- 19 Lepilina, A. *et al.* A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. *Cell* **127**, 607-619, doi:S0092-8674(06)01280-3 [pii] 10.1016/j.cell.2006.08.052 (2006).
- 20 Mercola, M., Ruiz-Lozano, P. & Schneider, M. D. Cardiac muscle regeneration: lessons from development. *Genes & Development* **25**, 299-309, doi:10.1101/gad.2018411 (2011).
- 21 Bersell, K., Arab, S., Haring, B. & Kuhn, B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* **138**, 257-270, doi:S0092-8674(09)00522-4 [pii] 10.1016/j.cell.2009.04.060 (2009).
- 22 Chen, H. S., Kim, C. & Mercola, M. Electrophysiological challenges of cell-based myocardial repair. *Circulation* **120**, 2496-2508, doi:120/24/2496 [pii] 10.1161/CIRCULATIONAHA.107.751412 (2010).
- 23 Senyo, S. E. *et al.* Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* **493**, 433-436, doi:10.1038/nature11682 (2013).
- 24 Zhang, Y. *et al.* Dedifferentiation and proliferation of mammalian cardiomyocytes. *PLoS ONE* **5**, e12559, doi:10.1371/journal.pone.0012559 (2010).
- 25 Jopling, C. *et al.* Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464**, 606-609, doi:10.1038/nature08899 (2010).
- 26 Brown, R. A., Wiseman, M., Chuo, C. B., Cheema, U. & Nazhat, S. N. Ultrarapid engineering of biomimetic materials and tissues: Fabrication of nano- and microstructures by plastic compression. *Adv Funct Mater* **15**, 1762-1770, doi:DOI 10.1002/adfm.200500042 (2005).

Endnote

Acknowledgments

We thank Drs. Laurence M. Brill for spectrometry, Fabio Cerignolli for providing valuable cells, Scott Metzler and Paul Kim for help with imaging, and Parisha Shah for assistance in mouse and pig experiments. This work was supported by NIH grants to PRL (HL065484 and R01 HL086879); MM (HL113601, HL108176, P01 HL098053); P30 AR061303 and P30 CA030199 for shared services and by the California Institute for Regenerative Medicine (CIRM, RC1-00132) to MM. KW and CH were SBMRI CIRM postdoctoral fellows (TG2-0116). VS was an Oak Foundation postdoctoral fellow. Support was also provided by NIH/NHLBI 5UM1 HL113456 to PCY; HL116591 to KWalsh, MJB was supported by the NIH (K08 AI079268) and the Stanford BioX Interdisciplinary Initiatives Program and NSF NSEC(PHY-0830228). Bin Zhou was supported by the National Basic Research Program of China (2013CB945302 and 2012CB945102) and the National Natural Science Foundation of China (91339104, 31271552, and 31222038). A Seed Grant to PRL from the Stanford Cardiovascular Institute supported the swine study.

Author Contributions

KW and WC performed experiments on EMCs and mESCs. KW generated mCMs^{ESC}, and performed cardiomyogenic and proliferation assays on mCMs^{ESC}, proliferation assays on NRVC. KW and WZ performed Mass Spectrometry experiments. MDC performed immunostaining of Fstl1. KW and AS performed calcium transient experiment. VS generated cardiac patch. VS, AW, and MJB performed biomechanical analysis of cardiac patch biomaterial. MZ and VS performed mouse MI experiments and echocardiography. YM, and PY performed MRI analysis.. KW and WC performed immunostaining of α -actinin, pH3 and aurora B. KW performed GFP, TUNEL and f4/80 staining.. MMh analyzed the release of Fstl1 from the patch *in vitro*. KWalsh, NK and SM performed experiments with Fstl1-TG mice. BZ performed experiments with adult mouse epicardium-conditioned media. KWalsh and SM provided data on systemic delivery of FSTL1. MN and MDS provided data on myocyte progenitors. DB supervised and coordinated in vivo mouse physiology experiments. VS, YM, and PY conducted the preclinical swine study. CH performed

FSTL1 overexpression and western blot experiments. KW,VS,MM and PRL designed experiments and prepared the manuscript.

Author Information

Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to PRL (prlozano@stanford.edu)

Figure Legends

Figure 1. Epicardial secretome contain cardiogenic activity, and improves cardiac function after MI via embryonic epicardium-like patches

a-d) Co-culture of mCMs^{ESC} cardiomyocytes with epicardial EMC cells. Representative micrographs (**a,b**). Quantification of myocyte number (**c**) and cardiac gene expression (**d**). *: $p < 0.05$ compared to acellular (EMC⁻) control; ■ $p < 0.05$ compared to 1×10^5 cells condition.

e-i) Culture of mCMs^{ESC} cardiomyocytes with EMC-conditioned media. Representative micrographs (**e,f**). Quantification of myocyte number (**g**), cardiac gene expression (**h**), and cardiomyocytes with rhythmic calcium transients (**i**). *: $p < 0.05$ compared to control.

j-m) Effect of adult epicardial media on embryonic cardiomyocytes from E12.5 GFP⁺ cells (*Tnnt2-Cre; Rosa26^{mTmG/+}*) (**j**). Conditioned media obtained from adult epicardial-derived cells (EPDCs) promotes cardiomyocyte proliferation that can be heat-inactivated (**k**) and cytokinesis analyzed by double immunostaining for Aurora B and Tnnt2 (cardiomyocytes) (**l, m**). *: $p < 0.05$

n) Cartoon of collagen patch generation (reconstructed from ²⁶).

o) Evaluation of mechanical properties of engineered patch, measured by atomic force microscopy.

p-q) Suture procedure of patch over ischemic myocardium.

r) Echocardiography analysis normalized to individual pre-surgery baseline values, **s) absolute values of fractional shortening (FS%)**, and **t) Masson's trichrome staining of the animal cohorts**: sham (control, n=10), infarcted mice without treatment (MI-only, n=8), MI treated with patch-only (MI+Patch, n=8), and infarcted animals treated with patch laden with epicardial conditioned media (MI+Patch+CM, n=8), 2 weeks after MI. *: $p < 0.05$ compared to Sham control, ●: $p < 0.05$ compared to MI-only, and ■: $p < 0.05$ compared to MI+Patch. (See **Methods** for details.)

Figure 2. Fstl1 is an epicardial cardiogenic factor with dynamic expression after ischemic injury.

a) MS/MS spectrum of Fstl1.

b-g) Fstl1 treatment of mCMs^{ESC} cardiomyocytes measured by: immunostaining (α -actinin, green) (**b,c**), quantification of myocyte number (**d**), expression of cardiac-specific markers (**e**), cardiomyocytes with rhythmic calcium transient (**f**), and individual cardiomyocyte cell size (**g**): statistically significantly different from control ($p < 0.05$).

h) Fstl1 immunostaining in the mouse embryonic heart (days E12.5, E15.5 and E17.5). Fstl1 (red), Wt1 (epicardial marker), α -actinin (myocardial marker), DAPI (nuclei). Fstl1 is expressed in epicardium (white arrowheads), no myocardium (yellow arrowheads).

i) Expression shift of Fstl1 in the mouse heart after MI. Trichrome staining (upper), labels fibrosis (blue) Fstl1 immunohistochemistry (lower panels, brown). Observe that in injured hearts Fstl1 expression is depleted from the epicardium (brown) and upregulated in the myocardium.

j-l) High resolution images of Fstl1 expression-shift after MI (see **Methods** for details).

Figure 3. FSTL1 recapitulates the *in vivo* restorative effect of epicardial conditioned-media in the engineered epicardial patch, and promotes cardiomyocyte proliferation.

a-b) Survival (**a**) and Kinetics of FS(%) (**b**) analyses after MI in the indicated treatments.

c) Effect of epicardial hFSTL1 patches on FS% in Fstl1-TG mice.

d-i) Masson's trichrome staining (**d**), morphometric analysis by echocardiography (**e**), and vascularization analysis (**f-i**) 4 weeks after MI. *: $p < 0.05$ compared to sham, ●: $p < 0.05$ vs MI-only, and ■: $p < 0.05$ vs MI+Patch.

j) Cross-sections covering infarct/patch area separated 250 μ m, 1-2mm from apex used for cardiomyocytes proliferation analysis (**k-p**), 4 weeks after MI.

k, m, o) Co-staining of pH3 and α -actinin (**k**), midbody-localized Aurora B kinase between α -actinin⁺ cells (**m**), and double-positive cells for pH3 and PCM1 (cardiomyocyte nuclei¹⁶) (**o**) 4 weeks post-MI, quantified in (**l, n, p**) normalized to myocardium area quantified by trichrome staining of immediate adjacent section. *: P<0.05 from sham. **: P<0.05 from all other groups.

q-v) Lineage tracing of FSTL1-responsive cells in 4-OH-tamoxifen treated *Myh6^{mERCremER};Rosa26^{Z/EG}* mice; patch+FSTL1 applied simultaneously to MI, and hearts were collected 4 weeks post-MI (**q**) with efficient labeling of cardiomyocytes (**r**). Infarcted hearts showing eGFP⁺ (pre-existing, green) cardiomyocytes positive for pH3 (yellow arrowheads) (white arrowheads: pH3⁺ eGFP⁻ cells) (**s-v**). (length of treatment (**a-c**) and n for each experiment indicated in graph, see **Methods** for details)

Figure 4. FSTL1 proliferative activity on early cardiomyocytes depends on cells- selective post-transcriptional FSTL1 modifications.

a-f) FSTL1 promotes proliferation of mCMs^{ESC}, measured by EdU incorporation (**a**), pH3 (**b**), and Aurora B immunostaining (**c**), and quantified in **d-f**.

g-i) Western blot analysis of Fstl1 secreted in cultured cardiomyocytes (myoFSTL1 CM) infected with Adeno-Fstl1 and in EMC (EMC CM) in the presence of tunicamycin (glycosylation inhibitor) (**g**), hFSTL1-V5 tagged expressed in AD-293 cells (**h**), and mammalian and bacterial-produced FSTL1 (**i**). Arrows: red: glycosylated; black: hypoglycosylated.

j) Mammalian-produced FSTL1 attenuates H₂O₂ induced apoptosis, while bacterial-produced FSTL1 cannot.

K, l) Bacterially-produced FSTL1 promotes mCMs^{ESC} EdU incorporation and Aurora B positivity whereas mammalian-produced FSTL1 does not.

m, n) Quantification of EdU incorporation in mCMs^{ESC} treated with conditioned media of EMC and Fstl1-overexpressing NRVC (concentration normalized to Fstl1 content).

*: statistically different from control, $P < 0.05$. (see **Methods** for details)

o) Working model of FSTL1 in distinct cardiac compartments.

Figure 5. Epicardial FSTL1 delivery activates cardiac regeneration in preclinical model of ischemic heart injury.

a-d) Time course MRI analysis of cardiac function in pigs. Functional analysis by measure of ejection fraction (EF%) (**a,b**). Scar size at week-4 post-grafting (**c,d**). Green lines highlight scar perimeter.

e-n) Analysis at week 4 post-grafting. Masson's trichrome staining (**e**). EdU incorporation (newly synthesized DNA) in the vascular smooth muscle cells (**f-h**). White line demarcates patch and host tissue. **i-m)** EdU incorporation and **n)** Aurora B kinase positivity in cardiomyocytes at week-4 post-grafting. (See **Methods** for details.)

Methods

Cell Preparation:

Progenitor cells $Sca1^+, Myh6^-$ cardiomyocyte progenitors were obtained by the Schneider laboratory as described¹⁸ (**Extended Data Fig. 7 k-m**).

Epicardial Mesothelial Cells (EMCs) were maintained in DMEM with 10% FBS and antibiotics/antimycotic as described²⁷. EMCs are stably transduced with H2B-mCherry Lentivirus for nuclei labeling (**Fig. 1, 4**).

Mouse embryonic stem cell-derived cardiomyocytes (mCMs^{ESC}): A stable mouse ESC line for drug resistance selection of cardiomyocytes ($Myh6-Puro^f; Rex-Blast^f$) was generated by lentiviral transduction and blasticidin selection, similarly to our previously reported human line²⁸

mCMs^{ESC} were obtained by differentiation of $Myh6-Puro^f; Rex-Blast^f$ mESCs in a differentiation media containing: Iscove's Modified Dulbecco Media (IMDM) supplemented with 10% FBS, 2mM glutamine, 4.5×10^{-4} M monothioglycerol, 0.5 mM ascorbic acid, 200 μ g/mL transferrin (Roche), 5% protein-free hybridoma media (PFHM-II, Invitrogen) and antibiotics/antimycotic as embryoid bodies (EBs) until day 4 and plated onto adherent cell culture plate until 9, one day after the onset of spontaneous beating. To purify $Myh6^+$ cardiomyocytes, puromycin was added at differentiation day 9 for 24 hours. Subsequently cells were trypsinized and plated as monolayer cardiomyocytes. Conditioned media and FSTL1 treatments were typically performed 24 hours after monolayer plating. The length of the treatments is indicated in each figure legends (**Fig. 1, 2, 4, Extended Data Fig. 1**).

AD-293 cells were directly purchased from Stratagene avoiding misidentification, and cultured in DMEM media with 10% FBS and with pen/strep. It's used for its high transfection efficiency and yield of recombinant proteins (**Fig. 4h**).

EMCs and $Myh6-Puro^f; Rex-Blast^f$ mESCs, and AD-293 cells are quarterly tested for mycoplasma contamination when in use.

Embryonic cardiomyocytes. We used fluorescence activated cell sorting (FACS) to purify cardiomyocytes from *Tnt-Cre;Rosa26^{mTmG/+}* (C57BL/6J and ICR mixed background) hearts from e12.5 embryos. Hearts were dissociated collagenase IV digestion and GFP⁺ cells for FACS purification. The GFP⁺ cells were cultured and confirmed to be cardiomyocytes by their expression of the cardiomyocyte specific markers alpha actinin (ACTN2) and cardiac troponin T (TNNT2). They were rhythmically beating when cultured *in vitro* (**Fig. 1j-m**).

Neonatal rat ventricular cardiomyocytes (NRVCs) were isolated with the neonatal rat cardiomyocyte isolation kit (Cellutron) and cultured at 37°C with 5% CO₂. In brief, ventricles were dissected from 1–2-d-old Hsd:SD rats (Sprague Dawley), then digested five times for 15 minutes each with the enzyme cocktail at 37°C. Cells were pooled, pre-plated for 90 minutes on an uncoated cell culture dish to remove fibroblasts, and plated on 1% gelatin-coated cell culture plastic dishes in high-serum media (DME/F12 [1:1], 0.2% BSA, 3 mM sodium-pyruvate, 0.1 mM ascorbic acid, 4 mg/liter transferrin, 2 mM L-glutamine, and 5 mg/liter ciprofloxacin supplemented with 10% horse serum and 5% fetal bovine serum (FBS)) at 3×10^5 cells/cm². After 24 hours, media was changed to low-serum medium (same but with 0.25% FCS) and cells cultured until use (**Fig. 4g, m, n, Extended Data Fig. 7g-j**).

Adult mouse cardiomyocytes were isolated from 3 mo old *Myh6^{mERCremER};Rosa26^{Z/EG}*. C57BL/6J mice as previously published²⁹. Briefly, mice were anesthetized with pentobarbital sodium (100 mg/kg IP). The heart was removed and retrograde perfused at 37°C with a Ca²⁺ free solution (in mM, 120 NaCl, 14.7 KCl, 0.6 KH₂PO₄, 0.6 Na₂HPO₄, 1.2 MgSO₄·7H₂O, 4.6 NaHCO₃, 10 Na-HEPES, 30 taurine, 10 BDM, 5.5 glucose) followed by enzymatic digestion with collagenase. Ventricles were cut into small pieces and further digested. Stop buffer (Ca²⁺ free solution + CaCl₂ 12.5 μM + 10% bovine calf serum) was added and the cell suspension was centrifuged at 40g for 3 min. Myocytes were resuspended in stop buffer in increasing CaCl₂ concentrations until 1mM was achieved. Cells were then resuspended in MEM + 5% bovine calf serum + 10mM BDM + 2mM L-Glutamine and added to the collagen solution, pre-polymerization (250,000 cells per ml or per patch). Following collagen gelation and plastic compression, cellular patches were cultured in aforementioned (plating) media overnight and then transferred into culture media: MEM + 1mg/ml bovine serum albumin + 25 μM blebbistatin + 2mM L-Glutamine, in presence or absence of recombinant FSTL1 (AVISCERA BIOSCIENCE, 10 ng/ml). At day 7, fluorescent ubiquitination-based cell-cycle indicator (FUCCI, Premo™ FUCCI Cell Cycle Sensor, Life Technologies, US) assay was conducted on the 3D culture specimens as previously described³⁰. In this assay, G1

and S/G2/M cells emit red and green fluorescence, respectively. The volume of Premo™ geminin-GFP and Premo™ Cdt1-RFP were calculated using the equation below:

$$\text{Volume of Premo™ geminin GFP or Premo™ Cdt1 RFP reagent (mL)} = \frac{\text{number of cells} \times \text{PPC}}{1 \times 10^8}$$

where the number of cells is the estimated total number of cells at the time of cell labeling (equal to CM seeding density, PPC (particles per cell) is the number of viral particles per cell (=40 in this assay), and 1×10^8 is the number of viral particles per mL of the reagent. The volumes of reagents calculated above were directly added to the cellular patches in complete cell medium, mixed gently, and incubated overnight in the culture incubator (≥ 16 hrs). Patch samples were imaged using a conventional fluorescence microscope, utilizing GFP and RFP filter sets (**Extended Data Fig. 7a-f**).

Co-culture experiments

mCMs^{ESC} are co-cultured with H2B-mCherry EMCs for 4 days and visualized by α -actinin immunofluorescent staining and H2B-mCherry fluorescence (**Fig. 1a, b**), cardiomyocyte counting (**Fig. 1c**, n=3), and cardiogenic gene expression normalized to *Gapdh* gene expression (**Fig. 1d** n=3)

Epicardial conditioned media:

Rat epicardial mesothelial cells (EMC) conditioned media. EMC²⁷ cells were cultured in 10% FBS DMEM with pen/strep until confluent ($\sim 1 \times 10^6/\text{cm}^2$), then washed with PBS 3 times and media is changed to serum free DMEM with pen/strep without phenol red and cultured for 2 additional days before the media was collected as conditioned media (20ml of media is added for conditioning and 18ml is collected after 2 days). Collected media was filtered through 0.22um pore membrane (Millipore). Control conditioned media were prepared the same way but without EMC cells (**Fig. 1e-l, n-t**).

Neonatal rat ventricular cardiomyocytes (NRVCs) conditioned media. NRVC were infected with adenovirus expressing un-tagged mouse Fstl1 at MOI 50. 24 hrs post-infection culture media was replaced by serum free media (DMEM/F12 with pen/strep). The media was conditioned with the infected NRVC and EMC cells for 24 hrs (**Fig. 4m, n**).

mCMs^{ESC} were treated with control and EMC-conditioned media for 8 days before α -actinin immunofluorescent staining (**Fig. 1e, f**), cardiomyocyte counting (**Fig. 1g**, n=3), analysis of cardiogenic gene expression normalized to *Gapdh* gene expression (**Fig. 1h** n=3) and quantification of the number of cardiomyocytes with rhythmic calcium transient measured automatically using a Kinetic Imaging Cytometer (Vala Sciences) (**Fig. 1i**, n=3).

mCMs^{ESC} were treated with serial dilutions of conditioned media of EMC and Fstl1-overexpressing NRVC for 24 hours with 10 μ g/ml EdU, and stained for α -actinin and EdU (**Fig. 4m, n**, n=5). The concentrations of the conditioned media are normalized to amount of Fstl1 expression by western blot.

Adult mouse EPDC conditioned media was generated in the Zhou laboratory⁸. Briefly, eight-week old adult *Wt1^{CreERT2/+};Rosa26^{mTmG/+}* hearts mice in C57BL/6J and ICR mixed background were injected orally 4 mg tamoxifen by gavage, four to five oral injections were administered during a two-week period. Myocardial infarction was then induced by ligation of left anterior descending coronary artery on (11 weeks old) adult mice. One week after injury, we collected *Wt1^{CreERT2/+};Rosa26^{mTmG/+}* hearts, which were then digested with collagenase IV into single cells. Digestion solution was made by adding 4ml 1% collagenase IV and 1ml 2.5% trypsin into 44.5 ml Hanks' Balanced salt solution, and supplemented with 0.5 ml chicken serum and 0.5 ml horse serum. Cells were re-suspended in Hank's balanced salt solution, 4ml digestion solution was added to each tube and rocked gently in 37°C shaker for 6 minutes. After removing the supernatant containing dissociated cells, we added another 4ml digestion solution to repeat the digestion 6 times. After final digestion, we filtrated the cells through 70 μ m filter and pellet cells by centrifuging at 200g for 5 minutes at 4°C. Cells were then re-suspended by Hanks' balanced salt solution for FACS isolation. Dissociated cells from GFP- hearts were used as a control for gate setting in FACS. GFP+ cells (epicardium-derived cells, EPDCs) were isolated from GFP+ *Wt1^{CreERT2/+};Rosa26^{mTmG/+}* hearts by FACS and these GFP+ purified populations were confirmed to be GFP+ cells under fluorescence microscope (**Fig. 1j**). FSTL1 expression (determined by PCR) was restored in cultured GFP⁺ EDPCs. Complete conditioned media from EPDCs was then added to embryonic cardiomyocytes culture for 48 hours before assay for proliferation (**Fig.1k-m**, n=5).

MTT Assays. Proliferation of cardiomyocytes treated with conditional medium was measured by MTT assay using Celltiter 96 Aqueous One solution (Promega) as previously described⁸. After

adding the Celltiter 96 Aqueous One reagent into the cell culture medium, we incubate the plate at 37°C for 3-4 hours, and then record the absorbance at 490nm using a 96-well plate reader. Absorbance at 490nm is tightly correlated with cell number. The MTT readout on the y-axis, labeled MTT assay (A490), thus reflects the relative number of cells from each well between groups of treatment (**Fig.1 k**). Boiling of conditioned media abolished the growth-promoting effects (**Fig.1 k**), suggesting a proteinaceous nature of the effective components.

Recombinant FSTL1 was purchased from AVISCERA BIOSCIENCE (00347-02-100, produced in E. Coli) and R&D system (1694-FN-050 , produced in mouse myeloma cell line, NS0-derived).

mCMs^{ESC} were treated with bacteria-synthesized recombinant human FSTL1 (10 ng/ml) for 8 days with media change every 2 days, before α -actinin immunofluorescent staining (**Fig. 2b, c**), cardiomyocyte counting (**Fig. 2d**, n=8), analysis of cardiogenic gene expression normalized to *Gapdh* gene expression (**Fig. 2e**, n=3) and quantification of the number of cardiomyocytes with rhythmic calcium transient measured automatically using a Kinetic Imaging Cytometer (Vala Sciences) (**Fig. 2f**, n=6). mCMs^{ESC} were treated with bacteria-synthesized recombinant human FSTL1 (6.25-50 ng/ml) for 2 days before measurement of individual cardiomyocyte cell size (in pixels) (**Fig. 2g**, n=5).

mCMs^{ESC} were stimulated with 6.25, 12.5, 25 and 50ng/ml of bacteria produced FSTL1 for 24 hours with 10 μ g/ml EdU, and stained for α -actinin and EdU (**Fig. 4a, d**, n=5). mCMs^{ESC} were stimulated with 10ng/ml FSTL1 for 48 hours and stained for α -actinin and pH3 (**Fig. 4b, e**, n=5). mCMs^{ESC} were stimulated with 25, 100, 200ng/ml FSTL1 for 48 hours, and stained for α -actinin (red) and Aurora B (**Fig. 4c, f**, n=5)

mCMs^{ESC} were stimulated with 10nM H₂O₂, and 10ng/ml bacteria and mammalian produced FSTL1 for 24 hours, and staining for α -actinin and TUNEL for cell death (**Fig. 4j**, n=5).

mCMs^{ESC} were stimulated with 10ng/ml of bacteria and mammalian produced FSTL1 for 24 hours with 10 μ g/ml EdU, and stained for α -actinin and EdU (**Fig. 4k**, n=5) , and α -actinin and Aurora B (**Fig. 4l**, n=5)

FSTL1 overexpression and western blot AD-293 cells were transiently transfected with human FSTL1 plasmid (GE Dharmacon, ID: ccsbBroad304_02639 pLX304-Blast-V5-FSTL1) using

lipofectamine 2000 (mocked transfection was done with lipofectamine and no plasmid). 48 hrs post-transfection serum containing media was replaced by serum free DMEM and incubated with the cells for 24 hrs. Tunicamycin was used at 2 μ g/ml. Conditioned media from tunicamycin samples was collected during 16 hrs (cells looked healthy). Conditioned media was spun at 400g 7 min and then concentrated approximately 20 times using Microcon-10 kDa cut off columns (Millipore). Samples were combined 1 to 1 ratio with 2x SDS sample buffer containing protease inhibitor, DTT and 5mM EDTA, boiled 10 minutes at 95C and run in a 4-15% acrylamide Mini-Protean TGX gel, transferred to nitrocellulose membrane and incubated with anti-V5 primary antibody MAB 15253 (Pierce) 1:1,000 dilution and anti-mouse 800 nm conjugated secondary antibody at 1:10,000 dilution (Odyssey), and scanned using Odyssey Clx Imager (**Fig. 4h**).

Neonatal rat ventricular cardiomyocytes were infected with adenovirus expressing un-tagged mouse Fstl1 at MOI 50. 24 hrs post-infection culture media was replaced by serum free media. Serum free DMEM/F12 pen/strep media was conditioned with the infected NRVC and EMC cells for 24 hrs. Tunicamycin was used at 1ug/ml and media was conditioned for 16 hrs. Conditioned media was spun at 400g 7 minutes and then concentrated using Microcon-10 kDa cut off columns (Millipore). Samples were combined 1 to 1 ratio with 2x SDS sample buffer containing protease inhibitor, DTT and 5mM EDTA, boiled 10 minutes at 95C and run in Any KD Mini-Protean TGX gel, transferred to nitrocellulose membrane and incubated with anti-FSTL1 MAB1694 (R&D) primary antibody 1:500 dilution and anti-rat 800 nm conjugated secondary antibody at 1:10,000 dilution (Odyssey), and scanned using Odyssey Clx Imager. Blocking and antibody incubation was done in Odyssey blocker. The western blot for recombinant FSTL1 (100ng each) was performed the same way (**Fig. 4g, i**).

RNA extraction and Q-RT-PCR . Total RNA was extracted with TRIzol (Invitrogen) and reverse transcribed to cDNA with QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. cDNA samples synthesized from 100ng of total RNA were subjected to RT-QPCR with LightCycler 480 SYBR Green I Master kit (Roche) performed with LightCycler 480 Real-Time PCR System (Roche) (**Fig. 1d, h, 2e, Extended Data Fig. b-d**). Primer sequences are listed in **Supplementary Table**:

LC-MS/MS analysis of conditioned-media. First, Tris(2-carboxyethyl)phosphine (TCEP) was added into 1 mL of conditional media to 10mM and the protein sample was reduced at 37°C for 30 min. Then iodoacetamide was added to 20mM and the solution was alkylated at 37°C for 40

min in the dark. Mass Spectrometry Grade of trypsin (Promega) was then added to the solution as 1:100 ratio. After overnight digestion at 37°C, the sample was then desalted using a SepPack cartridge, dried using a SpeedVac and re-suspended in 100 μ L of 5% formic acid. The resulting peptides were analyzed on-line by an LC-MSMS system, which consisted of a Michrom HPLC, a 15 cm Michrom Magic C18 column, a low flow ADVANCED Michrom MS source, and a LTQ-Orbitrap XL (Thermo Scientific, Waltham, MA). A 120-min gradient of 0-30%B (0.1% formic acid, 100% acetonitrile) was used to separate the peptides, and the total LC time was 141 min. The LTQ-Orbitrap XL was set to scan the precursors in the Orbitrap at a resolution of 60,000, followed by data-dependent MS/MS of the top 4 precursors.

The raw LC-MSMS data was then submitted to Sorcerer Enterprise (Sage-N Research Inc.) for protein identification against the IPI rat protein database, which contains semi-tryptic peptide sequences with the allowance of up to 2 missed cleavages and precursor mass tolerance of 50.0 ppm. A molecular mass of 57 Da was added to all cysteines to account for carboxyamidomethylation. Differential search includes 16 Da for methionine oxidation. The search results were viewed, sorted, filtered, and statically analyzed using PeptideProphet and ProteinProphet (ISB). The minimum trans-proteomic pipeline (TPP) probability score for proteins and peptides was set to 0.95, respectively, to assure TPP error rate of lower than 0.01. The example MS/MS spectrum R.GLCVDALIELSDENADWK.L was identified as Fstl1 (**Fig. 2a**). Peptide probability=1.0, Xcorr=6.276, delta Cn=0.471.

Automated *in vitro* cell proliferation and cell death assay: Cells (mCMs^{ESC} and NRVC) were incubated with EdU (details of dosage and length of exposure are specified in figure legends) in a 384 wells plate format, and were fixed for 2 hours in 4% PFA, washed in PBS and stained for EdU using Click-it EdU assay kit (Life Technologies). The cells were then washed in PBS, immunostained with an α -actinin antibody (Sigma, A7811, 1:500) to identify cardiomyocytes and stained with DAPI (4',6-diamidino-2-phenylindole, 1:10,000) to identify nuclei. The plates were then imaged using InCell 1000 system (GE Healthcare) and automatically analyzed in Developer Toolbox (GE Healthcare) as described³¹. Ratios of EdU⁺/ α -actinin⁺ nuclei and α -actinin⁺ nuclei were generated for the percentage of cardiomyocyte incorporated EdU in the chromosomal DNA.

Similarly, cells (mCMs^{ESC} and NRVC) in 384 wells plate format were fixed for 2 hours in 4% PFA, washed in PBS, and were immunostained with pH3 antibody (Millipore 06-570, 1:200) for nuclei in mitosis, or Aurora B (Millipore 04-1036, 1:200) for cytokinesis, or TUNEL (Roche) for cell death, and α -actinin antibody (Sigma, A7811, 1:500) for cardiomyocytes and DAPI (1:10,000) for nuclei.

The same imaging and analysis were done for pH3 staining as the EdU assays, and the Aurora B⁺, α -actinin⁺ double positive cells were manually counted. The percentages of pH3⁺, α -actinin⁺ double positive nuclei, Aurora B⁺, α -actinin⁺ double positive cells, and TUNEL⁺, α -actinin⁺ double positive nuclei relative to the total number of α -actinin⁺ cell nuclei were calculated to determine the percentages of cardiomyocytes undergoing mitosis, cytokinesis and apoptosis, respectively. Calcium Imaging. Contractile calcium transients were recorded using a Kinetic Image Cytometer (KIC, Vala Sciences) using Fluo4 NW calcium indicator (Life Science). Data was processed using Cyteseer software containing the KIC analysis package (Vala Sciences) as described³².

Compressed collagen gel for use as an engineered epicardial patch. Highly hydrated collagen gels – used as cardiac patch in this study – were produced by adding 1.1 ml 1X DMEM (Sigma, MO, US) to 0.9 ml of sterile rat tail type I collagen solution in acetic acid (3.84 mg/ml, Millipore, MA, US). The resulting 2 ml collagen-DMEM mixture was mixed well and neutralized with 0.1 M NaOH (~50 μ l). The entire process was conducted on ice to avoid premature gelation of collagen. In the case of patches containing epicardial factors, the EMC culture media was collected as above and 0.6 ml of that was mixed with 0.5 ml DMEM. The collagen solution (0.9 ml) was then distributed into the wells of 24-well plates (15.6 mm in diameter) and placed in a tissue culture incubator for 30 min at 37°C for polymerization. For pig studies, 6.8 ml of collagen was mixed with 8.2 ml DMEM to obtain a 15 ml solution that was then cast into a 6-cm Petri dish (A = 28.3 cm²). Plastic compression was performed as described previously^{33,34}. Briefly, as cast, highly hydrated collagen gels (at ~0.9 and 15 ml volumes for the mice and swine study, respectively) underwent unconfined compression via application of a static compressive stress of ~1,400 Pa for 5 minutes (see^{33,35} for details), resulting in ~98-99% volume reduction (**Fig. 1n**). The elastic modulus of the compressed collagen, aimed to approximate that of the embryonic epicardium which is optimal for contractility of immature cardiomyocytes (see text³⁶), was assessed by atomic force microscopy (AFM) in nano-indentation mode, using a force trigger that resulted in a minimal local strain of less than 10% (indentation of ~100 nm) to minimize the effect of substrate-related artifacts. A custom-made flat AFM tip was manufactured using focused ion beam milling and utilized to probe the stiffness of the gels by scanning areas of 90 μ m \times 90 μ m. Histogram of the distribution of measured micro stiffness of the patch is compared with the range of elasticity reported for common scaffolding biomaterials³⁷, and previously described³⁶ optimal range of elasticity to maximize myocyte contractility (**Fig. 1o**, n=3).

Myocardial Infarction and application of the epicardial patch

Permanent LAD occlusion (MI): Male 10-12 weeks old C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Fst11-TG mice used in MI experiments are C57BL6 background, female and male mice age 12-15 weeks old. Mice were anesthetized using an isoflurane inhalational chamber, endotracheally intubated using a 22-gauge angiocatheter (Becton, Dickinson Inc., Sandy, Utah) and connected to a small animal volume-control ventilator (Harvard Apparatus, Holliston, MA). A left thoracotomy was performed via the fourth intercostal space and the lungs retracted to expose the heart. After opening the pericardium, a 7-0 suture was placed to occlude the left anterior descending artery (LAD) ~2 mm below the edge of the left atrium. Ligation was considered successful when the LV wall turned pale (**Fig. 1p**). In the case of experimental groups treated with patch, immediately after the ligation, prepared collagen patch was sutured (at two points) onto the surface of ischemic myocardium (**Fig. 1g**). The patch size used was ~ one third of the 15.6 mm-diameter collagen gel. Animals were kept on a heating pad until they recovered. Another group of mice underwent sham ligation; they had a similar surgical procedure without LAD ligation. A minimum number of $n=8$ was used in each study group.

Ischemia reperfusion (I/R). Male C57/BL6, aged 10 to 11 weeks, were anesthetized and intubated as described above. A left lateral thoracotomy was then performed. Pericardium was gently pulled off and an 8-0 Nylon suture (Ethicon, Inc. Johnson & Johnson Co., USA) was used to ligate the left anterior descending coronary artery against a PE10 tubing, which was removed after 30 minutes occlusion. Successful performance of coronary artery occlusion was verified by visual inspection (by noting the development of a pale color in the distal myocardium upon ligation). The chest was then closed using 7-0 sutures around adjacent ribs, and the skin was closed with 6-0 suture. Buprenorphine was administered subcutaneously for a minimum of 1 day at BID dosing. For the animal group treated with patch, a second thoracotomy was performed one week post the incidence of I/R and the prepared collagen patch was sutured (at two points) onto the surface of ischemic myocardium. Sham-operated controls consisted of age-matched mice that underwent identical surgical procedures (two thoracotomies) with the exception of LAD ligation (**Extended Data Fig. 4**).

Echocardiography. In vivo heart function was evaluated by echocardiography at 2 weeks (**Fig. 1r, s, Fig. 3b, c, Extended Data Fig. 2 h-j**), 4 weeks (**Fig. 3b, c, e, Extended Data Fig. 2 h-j**), and 2 and 3 months (**Fig. 3b**) after LAD ligation. Two-dimensional (2D) analysis was performed on mice using a GE Vivid 7 ultrasound platform (GE Health Care, Milwaukee, WI) equipped with

13 MHz transducer. The mice were sedated with isoflurane (100 mg/kg, inhalation), and the chest was shaved. The mice were placed on a heated platform in the supine or left lateral decubitus position to facilitate echocardiography. 2D clips and M-mode images were recorded in a short axis view from the mid-left ventricle at the tips of the papillary muscles. LV internal diameter (LVID) and posterior wall thickness (LVPW) were measured both at end diastolic and systolic. Fractional shortening (FS, %) and ejection fraction (EF, %, via extrapolation of 2D data) were calculated from LV dimensions in the 2D short axis view. A minimum number (n) of 8 mice per experimental group was used for the echo evaluations. Measurements were performed by two independent groups in a blind manner. In ischemia reperfusion study, in vivo heart function was evaluated pre-surgery (baseline), 1 week after the incidence of I/R, and two and four weeks post-implantation (**Extended Data Fig. 4a-d**).

***In vivo* delayed-enhanced magnetic resonance imaging (DEMRI).** To prepare for scanning, induction of anesthesia was accomplished with 2% and maintained with 1.25-1.5% isoflurane with monitoring of the respiratory rate. ECG leads were inserted subcutaneously to monitor the heart rate while the body temperature was maintained at 37°C. Using 3T GE Signa Excite clinical scanner with a dedicated mouse coil (Rapid MR International, Germany), functional parameters were recorded on weeks 1 and 4 after treatment. The following sequences were performed for MRI acquisitions: (1) DEMRI was performed following IP injection of 0.2mmol/kg gadopentetate dimeglumine (Magnevist, Berlex Laboratories) using gated fGRE-IR sequences with FOV 3.4cm, slice thickness 0.9mm, matrix 128x128, TE 5ms, TI 150-240ms, and FA 60°; and (2) cardiac MRI of volumes were performed using fSPGR with FOV 7cm, slice thickness 0.9 mm, matrix 256x256, TE 5.5ms, and FA 30. Coronal and axial scout images were used to position a 2-dimensional imaging plane along the short axis of the left ventricular (LV) cavity (**Extended Data Figure h-j**).

Histology, Immunohistochemistry and Immunofluorescent staining Histological analysis (Mason's trichrome staining) was performed according to standard protocols for paraffin embedded samples. For immunohistochemistry and immunofluorescent staining, embedded hearts were sectioned at a thickness of 7 µm, unless described otherwise. Antibodies used were as follows: 1:200 α-actinin (Sigma, A7811), 1:300 α-smooth muscle actin (Sigma A2547) 1:100 phospho-Histone3 (rabbit Millipore 06-570), 1:300 phospho-Histone3 (mouse Abcam ab14955) 1:100 WT1 (Abcam, ab15249), 1:100 (**Fig. 1l-m**) and 1:250 (**Fig. 3m, n, Fig. 5n**) Aurora B (Millipore 04-1036), 1:100 Tnnt2 (DSHB, Ct3), 1:100 Tnni3 (Abcam, ab56357), 1:200 PCM1 (Sigma-Aldrich HPA023370), 1:200 FSTL1 (R&D MAB17381). At least 5 sections per heart were used per staining for Mason's Trichrome staining and 3 sections per heart per staining for

immunohistochemistry and immunofluorescent staining, respectively. HRP Anti-rat secondary antibody (Jackson ImmunoResearch 712-036-153, 1:500) was used for immunohistochemistry, and respective fluorescent secondary antibodies (Life Technologies 1:200) were used for immunofluorescent staining. The Trichrome staining and immunohistochemistry images were taken using an upright Zeiss microscope and dissection scopes. The fluorescent images were taken using Apotome Optical Sectioning (Zeiss). An inclusion criterion for the patch engraftment was that the patch covered > 70% of the infarct (controlled by histology). TUNEL assay (Roche 11684795910), and EdU assay (Life Technologies C10337) were performed as instructed.

Lineage tracing experiments

Epicardial lineage labeling was achieved by oral delivery of tamoxifen (4 mg) in eight-week old *Wt1^{CreERT2/+};Rosa26^{RFP/+}* mice with C57BL/6J and ICR mixed background (delivered 6 times for duration of 3 weeks and stopped 1 week before MI). Hearts were collected at 2 weeks after MI. Immunostaining of RFP for *Wt1* lineage cells, *Fstl1* and *Tnni3* shows that *Fstl1* is absent in epicardial cells and their derivatives, but abundant in the myocardium after MI (**Fig. 2I**).

Cardiomyocyte lineage labeling was achieved by injecting 4-OH tamoxifen intraperitoneally into eight-week old *Myh6^{mERCremER};Rosa26^{Z/EG}* mice¹⁷ of C57BL6 background at a dose of 20 mg/kg/d for 2 weeks, and stopped 1 week before harvesting cardiomyocytes (**Extended Fig. 7a-f**), or MI operation and patch grafting. 4 weeks after MI, the animals were collect for immunostaining (**Fig. 3q-v**).

TTC staining At day 2 post MI/patch treatment, the mouse hearts from all four groups were harvested and sectioned perpendicularly to the long axis into four sections (approximately 2 mm thick). The sections were placed in the wells of a 12-well cell culture plate and incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) solution for 15 mins at 37°C. Subsequently section were washed with PBS and visualized using a stereomicroscope and photographed with a digital camera (**Extended Data Figure 6a, b**).

Vessel counting. Blood vessel density parameters were measured from histological sections of heart samples stained for von Willebrand factor (vWF) as a marker of endothelial cells in the vessel wall. Up to 60 sections were analyzed for each treatment group (4 mice in each group). Analysis was performed using ImageJ to calculate: 1) the total luminal area of blood vessels, and

2) the number of vessels that stained + for the vWF. In each case, a histogram of the vessel parameters as a fraction of total surface area analyzed was obtained and the mid-values plotted for each treatment group. Statistical significance ($p < 0.05$) of the differences from sham group was determined by one-tailed ANOVA (**Fig. 3f-i**).

Cardiomyocyte proliferation quantification *in vivo*. Data collected from 5-7 hearts in each group (7 for MI+Patch+FSTL1, 5 for Sham, MI-only and MI+Patch) with 3 different cross sections (each section covered the infarct, patch, and separated by 250 μ m, between 1-2mm from the apex) counted exhaustively for total pH3⁺/ α -actinin⁺, Aurora B⁺/ α -actinin⁺, and pH3⁺/PCN1⁺ cells in each section, and normalized to myocardium area quantified by trichrome staining of immediate adjacent section (**Fig 5j-p**, and **Extended Data Fig 5**). .

Enzyme-linked Immunosorbent Assay. In order to assess the FSTL1 retention within the engineered patch system *in vitro*, collagen scaffolds laden with FSTL1 (5 μ g/ml) were immersed in PBS and shaken for various times (0, 12 hours, 1 day, and 21 days) at 37°C and the FSTL1 concentration was determined using Enzyme-linked Immunosorbent Assay kit (USCN Life Science, Inc., Houston, USA). The detection limit for this technique was 0.50 ng/ml. Scaffolds were pretreated with 1 mg/ml collagenase type I (Sigma Aldrich, MO, US) and 5 mg/ml hyaluronidase (Sigma Aldrich, MO, US) dissolved in phosphate buffered saline for 5 minutes followed by centrifugation at 5,000 \times g for 20 minutes.

Aliquots of 100 μ l of the collected samples were added to the 96-well plates and incubated for 2 hours at 37°C. Then, 100 μ L of the prepared detection reagent A were added to the wells followed by 1 hour incubation at same temperature. After aspiration and washing 3 times, 100 μ l of the prepared detection reagent B was added to the wells and incubated for 30 minutes at 37°C. After aspiration and washing 5 times, 90 μ L of substrate solution was added to the wells following by incubation for 25 minutes at 37°C. 50 μ L of stopping solution was added to the wells and the absorbance of each well was read at 450 nm, immediately. The concentration of FSTL1 was defined using standard curve of the standard solutions. The test was performed 4 times (**Extended Data Fig. 3a**).

Application of the patch in a swine model of ischemia-reperfusion. The swine study was performed by inflation of a percutaneous coronary angioplasty dilation catheter to occlude the LAD in Yorkshire pigs (45 days old). Occlusion time of 90 mins was followed by fully reperfusion to mimic the clinical MI disease model. One week after MI, a left thoracotomy was performed and

the patch (6-cm diameter) was sutured onto the infarct. Animal groups included: sham controls, I/R with no treatment (n=3), I/R treated with patch alone (I/R+Patch, n=1), and I/R treated with patch laden with FSTL1 (I/R+Patch+FSTL1, n=2). EdU delivery: 250 mg/week EdU was infused into circulation during the 4-week time course of study (week 1 to week 5 post I/R), using osmotic mini pumps (**fig. 5**).

Animal Compliance

The procedures involving animal use and surgeries were approved by the Stanford Institutional Animal Care and Use Committee (IACUC). Animal care and interventions were provided in accordance with the Laboratory Animal Welfare Act (C57BL/6J wildtype mice (**Fig. 1p-t, Fig. 2h-k, Fig. 3a-p, Extended Data Fig. 3-6**) *Myh6^{mERCremER};Rosa26^{Z/EG}*. C57BL/6J mice (**Fig. 3q-v, Extended Data Fig. 7a-f**) Yorkshire pigs (**Fig. 5**)).

The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Boston University (wildtype and Fstl1-TG C57BL/6J mice, (**Extended Data Fig. 2, Fig. 3c**)).

Mice were used in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (*Tnt-Cre;Rosa26^{mTmG/+}*, *Wt1^{CreERT2/+};Rosa26^{mTmG/+}*. mice (C57BL/6J and ICR mixed background) (**Fig. 1j-m**) *Wt1^{CreERT2/+};Rosa26^{RFP/+}* mice (C57BL/6J and ICR mixed background) (**Fig. 2l**)

All animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of Sanford-Burnham-Prebys Medical Discovery Institute. All animal procedures performed conform the NIH guidelines (Hsd:SD rats (**Fig. 4g, m, n, Extended Data Fig. 7g-j**)).

Statistical analysis The number of samples (*n*) used in each experiment is recorded in the text and shown in figures. All *in vitro* experiments have been done at least twice independently. Gene expression experiments have been done 3 times independently and EdU proliferation assays and cell size measurement have been done more than 10 times independently. Sample size was not pre-determined, with retrospective analysis of significantly different results in most *in vitro* studies using Gpower 3.1 produces power > 0.8. Sample sizes for animal studies were estimated. Animals which did not survive up to 4 weeks after surgery were excluded from functional and histological studies. Randomization was not applied. Blinding to group allocation was practiced between animals surgery and results analysis of mouse myocardial infarction experiments. The values presented are expressed as means ± SEM. The rationale to use means ± SEM instead of SD is that SEM quantifies uncertainty in an estimate of the mean whereas SD indicates dispersion

of the data from mean. In other words, the SEM provides an estimate of the reported mean value, while the SD gives an idea of the variability of single observations. Normal distribution were tested and confirmed in automatic analysis of mCms^{ESC} (Fig. 1c, g, i, Fig. 2d, f, g, Fig. 4d, e, j, k, l, m, n, Extended Data Fig. 1, 7h, j). We did not estimate variations in the data. The variances are similar between the groups that are being statistically compared. One-way ANOVA with multiple comparisons (Fig. 1r, 3, Extended Data Fig. 2b, d, 4, 5, 6) and student T-test (Fig. 1a-m, 2, 4, Extended Data Fig. 2e-n, 7) were used to test for statistical significance ($P < 0.05$). Survival curve were generated by Kaplan-Meier method using PRISM (GraphPad) and Log-rank (Mantel-Cox) test was used to test the significant differences between the survival of mice in different conditions (Fig. 3a).

Methods References

- 27 Eid, H. *et al.* Role of epicardial mesothelial cells in the modification of phenotype and function of adult rat ventricular myocytes in primary coculture. *Circ Res* **71**, 40-50 (1992).
- 28 Kita-Matsuo, H. *et al.* Lentiviral vectors and protocols for creation of stable hESC lines for fluorescent tracking and drug resistance selection of cardiomyocytes. *PLoS ONE* **4**, e5046, doi:10.1371/journal.pone.0005046 (2009).
- 29 Fajardo, G. *et al.* Deletion of the beta2-adrenergic receptor prevents the development of cardiomyopathy in mice. *J Mol Cell Cardiol* **63**, 155-164, doi:10.1016/j.yjmcc.2013.07.016 S0022-2828(13)00249-6 [pii] (2013).
- 30 Sakaue-Sawano, A. *et al.* Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* **132**, 487-498, doi:10.1016/j.cell.2007.12.033 S0092-8674(08)00054-8 [pii] (2008).
- 31 Bushway, P. J. & Mercola, M. High-throughput screening for modulators of stem cell differentiation. *Methods Enzymol* **414**, 300-316, doi:S0076-6879(06)14017-3 [pii] 10.1016/S0076-6879(06)14017-3 (2006).
- 32 Cerignoli, F. *et al.* High Throughput Drug Risk Assessment in Human Cardiomyocytes by Kinetic Image Cytometry. *Submitted (J. Pharm. Toxicol. Methods)* (2012).
- 33 Serpooshan, V. *et al.* Reduced hydraulic permeability of three-dimensional collagen scaffolds attenuates gel contraction and promotes the growth and differentiation of mesenchymal stem cells. *Acta Biomater* **6**, 3978-3987, doi:S1742-7061(10)00217-5 [pii] 10.1016/j.actbio.2010.04.028 (2010).
- 34 Serpooshan, V., Muja, N., Marelli, B. & Nazhat, S. N. Fibroblast contractility and growth in plastic compressed collagen gel scaffolds with microstructures correlated with hydraulic permeability. *J Biomed Mater Res A* **96**, 609-620, doi:10.1002/jbm.a.33008 (2011).
- 35 Abou Neel, E. A., Cheema, U., Knowles, J. C., Brown, R. A. & Nazhat, S. N. Use of multiple unconfined compression for control of collagen gel scaffold density and mechanical properties. *Soft Matter* **2**, 986-992, doi:Doi 10.1039/B609784g (2006).

- 36 Engler, A. J. *et al.* Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. *J Cell Sci* **121**, 3794-3802, doi:10.1242/jcs.029678 (2008).
- 37 Venugopal, J. R. *et al.* Biomaterial strategies for alleviation of myocardial infarction. *Journal of the Royal Society, Interface / the Royal Society* **9**, 1-19, doi:10.1098/rsif.2011.0301 (2012).
- 38 Clement, S. *et al.* Expression and function of alpha-smooth muscle actin during embryonic-stem-cell-derived cardiomyocyte differentiation. *J Cell Sci* **120**, 229-238, doi:jcs.03340 [pii]
10.1242/jcs.03340 (2007).

EXTENDED DATA

Wei, Serpooshan et al.

Epicardial FSTL1 Reconstitution Regenerates the Adult Mammalian Heart

Contents:

- **Extended Data Figure Legends**
- **Extended Data Tables**

Extended Data Figure Legends

Extended Data Figure 1. Characterization of mCMs^{ESC} cells used in this study.

a) Schematic time-line of cell preparation and treatment.

b-d) Immunostaining of α -actinin of mCMs^{ESC}, showing that the majority of the cells are α -actinin⁺ (**b**), and the α -actinin lacks striation structures (**c**). **d)** Immunostaining of α -smooth muscle actin (α SMA) of mCMs^{ESC}, showing the majority of the cells are α SMA⁺, unlike mature cardiomyocytes with no SMA expression³⁸.

e-f) Automatic detection of EdU incorporation in mCMs^{ESC}. Captured image of mCMs^{ESC} treated with 10 μ g/ml EdU for 24 hours, stained with EdU, α -actinin and DAPI using InCell 1000 (General Electric) (**e**). Overlay of masks of EdU, α -actinin and DAPI channels with automatic detection software (**f**).

g) EdU incorporation profile of mCMs^{ESC} over time. mCMs^{ESC} are treated with 10 μ g/ml EdU for 24 hours at time 0 hour, 24 hours, 48 hours, and 144 hours. The percentage of EdU⁺/ α -actinin⁺ cardiomyocytes of all α -actinin⁺ cardiomyocytes is calculated for each time period. Note the decrease of EdU incorporation rate over time.

h,i) Fluo 4 calcium images of mCMs^{ESC}, with baseline background image (**h**) and peak image (**i**).

j) Comparison of representative calcium transients of mCMs^{ESC} (red) and neonatal rat ventricular cardiomyocytes (NRVC, blue). Note the reduced amplitude, slower rate of up and down strokes, and elongated duration of the calcium transient in mCMs^{ESC} compared to NRVC, suggesting immature calcium handling in mCMs^{ESC}.

In all experiments, FSTL1 was added one day after plating of the mCMs^{ESC} (time 0-24 in this figure).

Extended Data Figure 2. Myocardial overexpression of Fstl1 (Fstl1-TG) mice after permanent LAD ligation.

a-d) Fstl1 protein expression kinetics after myocardial infarction. Fstl1-TG mice (C57/Bl6 background) and littermate wild type (WT) mice underwent LAD ligation. Heart tissue and serum were collected at baseline, day 1, day 3, day 7 and day 28 after surgery. Fstl1 protein levels in ischemic area (IA) and remote area (RM) of heart were analyzed by Western blotting (**a**). Fstl1 expression expressed relative to tubulin levels is reported (**b**). Fstl1 serum levels were analyzed by Western blotting (**c**). Also shown in Ponceau-S staining to indicate equal loading of serum. Quantification of serum Fstl1 level is shown in (**d**). $n > 3$ in all groups. *: $P < 0.05$ compared to WT baseline, #: $P < 0.05$ compared to Fstl1-TG baseline. ANOVA was used for statistical significance ($P < 0.05$).

e-j) Morphometric and functional response of Fstl1-TG mice to permanent LAD ligation at long-term. Representative Masson's trichrome staining of WT (**e**) and Fstl1-TG (**f**) 4 weeks after MI. Quantification of content in fibrotic tissue at week 4 after MI (**g**). Echocardiographic measurement of left ventricular internal dimension in systole (LVIDs) (**h**), and left ventricular internal diameter in diastole (LVIDd) (**i**) at weeks 2, 4 after MI. Echocardiographic determination of fractional shortening (FS%) in the indicated genotypes at 2 and 4 weeks after MI (**j**).

(k-n) Double immunofluorescent staining of α -actinin (cardiomyocytes) and pH3 (mitosis) (**k**) and α -actinin (cardiomyocytes) and von Willebrand factor (vascular endothelial cells) (**m**) in the Fstl1-TG and WT mice, quantified in (**l, n**). $n = 5$, *, significantly different ($P < 0.05$) from WT.

Extended Data Figure 3. Patch+FSTL1 attenuated fibrosis after MI

a-f) FSTL1 retention in the patch *in vitro* and *in vivo*. **a,b)** Enzyme-linked immunosorbent assay used to measure the amount of FSTL1 retained within collagen scaffolds exposed to PBS ***in vitro* for different time intervals (0-21 days) (a).** **The Table lists the initial and final FSTL1 concentration, as well as the release values within the first 24 hours (b).** **c-f)** FSTL1 retention in the patch *in vivo*. Representative images of Fstl1 immunostaining in the indicated animal treatment groups, week 4 after surgery. Note that, while Fstl1 is expressed in the uninjured epicardium (arrow in the inset in **c**), its expression became undetectable within the infarct area after MI (**d**). Similarly, no FSTL1 was detected in the MI+Patch animals (**e**), while it still persists (red staining) in the patch area of the MI+Patch+FSTL1 group (**f**).

g) Representative Masson's Trichrome staining on serial cross sections of hearts under 4 conditions (sham, MI only, MI+patch and MI+patch+FSTL1) 4 weeks after MI. Note the severe fibrosis in MI only condition, and reduced fibrosis in MI+Patch condition, and further reduction in

MI+Patch+FSTL1 condition, quantified in **Fig. 3d**.

h-j) Representative MRI images from the mouse MI-only, MI+patch and MI+Patch+FSTL1 treatment groups showing the 3D-FSPGR (fast spoiled gradient-echo) images and the delayed enhancement images utilizing gadolinium contrasting agents, confirming a reduction in infarct area (demarcated in green) and preserved contractility (**Suppl. Videos 3-5**).

k) Trichrome staining of infarct and border zone of the indicated treatments demonstrates the integration of the patch with the host tissue and massive patch cellularization by the native cardiac cells. Observe the abundant muscle (red) inside the patch and in the border zone of the patch+FSTL1 treated animals (three right panels, green arrowheads).

Extended Data Figure 4. Analysis of patch+FSTL1 function in the mouse model of ischemia/reperfusion (I/R) with delayed patch grafting.

a-c) Heart function evaluation for sham, I/R, and I/R treated with patch+FSTL1, at end- diastolic and systolic, pre-grafting (**a**, 1 week post injury), 2 weeks post patch implantation (**b**), and 4 weeks post grafting (**c**). Values were normalized by dividing to pre-surgery baseline values for each individual animal. **d)** Absolute values of fractional shortening (FS, %) at different times pre and post I/R as evaluated by echocardiography of mice from **a-c**. Abbreviations same as in **Fig. 3**. *: $p < 0.05$ compared to sham and •: $p < 0.05$ compared to I/R. **e)** co-immunofluorescence staining of DNA duplication marker phospho-Histone3 Ser10 (pH3, green) and α -actinin (red) in the border zone of Patch+FSTL1 treated heart 4 weeks after MI. **f)** Quantification of incidence of pH3⁺, α -actinin⁺ double positive cells in the 3 experimental groups. Data collected from 3 hearts in each group with 3 different cross sections counted for total pH3⁺, α -actinin⁺ cells in each heart. *: statistically different from all other groups, $P < 0.05$.

Extended Data Figure 5. Representative images and quantification of cardiomyocyte proliferation *in vivo* after Patch+FSTL1 treatment

a-h) Immunostaining of the cardiomyocyte marker α -actinin (red) in the infarct area (**b-d**) and co-immunofluorescence staining of DNA duplication marker phospho-Histone3 Ser10 (pH3, green) and α -actinin (red) in the border zone (**f-h**), in the 4 treatment groups analyzed 4 weeks post-MI,

compared to sham-operated animals (**a,e**). Insets in (**a-d**) show lower magnification images with broken lines demarcating the border between the patch and host tissues. Arrowheads in (**g,h**) indicate α -actinin⁺ cardiomyocytes with pH3⁺ nuclei,.

i-k) Representative images of pH3⁺ cardiomyocytes in a Patch+FSTL1 treated heart. Masson's Trichrome staining of a heart after MI 4 weeks treated with Patch+FSTL1 (**i**). The adjacent slide was stained for α -actinin in (**j**), corresponding to the black box area with infarction and the patch in (**i**). The spotted line in (**j**) indicates the boundary between the heart and the patch. The adjacent slide was stained for α -actinin and pH3, and all α -actinin⁺, pH3⁺ double positive cardiomyocytes found were shown in (**k**) (white arrowhead), with each image corresponding to the area in numbered white boxes in (**j**).

l-n) Quantification of cardiomyocyte proliferation measured in 3 cross sections covering the infarct, patch, and separated by 250 μ m, between 1-2mm from the apex in each heart (**Fig. 3j**). Data collected from 5-7 hearts in each group with the 3 cross sections counted exhaustively for incidence of α -actinin⁺ cells positive for pH3 (**l**), midbody-localized Aurora B kinase between α -actinin⁺ cells (**m**), and double-positive cells for pH3 and the nuclear cardiomyocyte maker PCM1 (**n**), and normalized to myocardium area quantified by trichrome staining of immediate adjacent section. *: statistically different from sham, P<0.05. **: statistically different from all other groups, P<0.05.

o) Quantification of hypertrophy in all experimental groups, measured by counting cardiomyocytes in areas of intraventricular wall with perpendicular cross sections of cardiomyocytes in all hearts analyzed for cardiomyocyte proliferation. No significance were found between samples.

p-r) Quantification of incidence of α -actinin⁺ cells positive for pH3 (**p**), midbody-localized Aurora B kinase between α -actinin⁺ cells (**q**), and double-positive cells for pH3 and the nuclear cardiomyocyte maker PCM1 (**r**) measured in **l-m** to total number of cardiomyocytes, calculated using hypertrophic analysis results in **o**. *: statistically different from sham, P<0.05. **: statistically different from all other groups, P<0.05.

s, t) Quantification of incidence of α -actinin⁺ cells positive for pH3 (**s**) and midbody-localized Aurora B kinase between α -actinin⁺ cells (**t**), separated by their localization in the border zone or infarcted area. Note the majority of proliferation quantified by both methods are located in the border zone, *: statistically different from all other groups, P<0.05.

Extended Data Figure 6. Effect of implantation of patch+FSTL1 on apoptosis and inflammation.

a) Representative TTC staining of day 2 post MI/patch treatment of all four groups (sham, MI, MI+Patch, MI+Patch+FSTL1). **b)** Quantification of area at risk comparing all 4 groups. Data collected from 4 hearts in each group, with 4 cross sections, approximately 2 mm thick each, encompassing each heart. *: statistically different from the sham, $P < 0.05$.

c, d) Representative image of TUNEL assays (TUNEL, green, α -actinin, red) comparing hearts 2 days after MI with patch alone and patch+FSTL1. **e)** Quantification of TUNEL⁺, α -actinin⁺ in infarcted area, as percentage of total number of cardiomyocyte. No difference is observed between MI+Patch and MI+Patch+FSTL1 conditions. Data collected from 3 hearts in each group with 3 different cross sections (same as in **Fig. 3j**) Ten 0.09mm² images were taken from infarcted area of each section and counted for TUNEL⁺, α -actinin⁺ and total α -actinin⁺ cells.

f-j) TUNEL staining for cell death and α -actinin staining for cardiomyocytes were performed on hearts treated with patch-only and patch+FSTL1 at day 4 and day 8 after MI (**f-i**). Minimal TUNEL⁺, α -actinin⁺ cells are detected while there are significant amount of TUNEL⁺, α -actinin⁻ cells. Quantification of all TUNEL⁺ nuclei showed no significant differences between Patch and Patch+FSTL1 treated hearts at both time points (**j**).

k-o) Immunostaining of F4/80 for macrophages and α -actinin for cardiomyocyte were performed on the same hearts as in panels a-d (**k-n**). Quantification of F4/80⁺ cells showed no significant differences between Patch and Patch+FSTL1 treated hearts at both time points (**o**).

Extended Data Figure 7. FSTL1 does not induce proliferation in adult and neonatal cardiomyocytes, or cardiac progenitor cells.

a-f) Adult cardiomyocytes derived from mouse primary isolation. **a)** Visualization of GFP⁺ cardiomyocytes isolated from *Myh6*^{mERCremER};*Rosa26*^{Z/EG} mice treated with 4-OH-tamoxifen (OH-Tam) in 3D-collagen patches. **b-d)** Gene expression changes in adult cardiomyocyte treated with FSTL1, including proliferation (**b**), cardiac-specific (**c**), and hypertrophy (**d**) markers. Note no changes in expression of cardiac specific genes, no increase in cell cycle markers (consistent with undetectable Ki67 immunostaining), and decreased hypertrophy markers (n=3). Cardiomyocytes were embedded within 3D patch were treated with FSTL1 (10ng/ml) for duration of 7 days with media change every 2 days.

e,f) FUCCI assay in 3D-cultured adult cardiomyocytes, conducted 1 week after the 3D culture. **e)** Treatment with FSTL1 was performed for 7 days with media change every 2 days. **f)** Adult cardiomyocytes 3D-cultured control in absence of FSTL1. Note no detectable sign of cardiomyocytes in S/G2/M phases (GFP⁺) in either condition. Purple arrows point to purple-colored nuclei resulting from co-localization of Hoechst (blue) and G1 phase FUCCI (red) labeling. **(g-j) Primary neonatal rat ventricular cardiomyocytes (NRVC).** **g,h)** Freshly isolated NRVCs stimulated with FSTL1 for **48 hours** with 10µg/ml EdU, and stained for α -actinin (red) and EdU (green). Percentages of EdU⁺/ α -actinin⁺ cardiomyocytes of all α -actinin⁺ cardiomyocytes are quantified (**h**). **i, j)** NRVCs stimulated with FSTL1 for 48 hours, and stained for α -actinin (red) and pH3 (green). Percentages of pH3⁺/ α -actinin⁺ cardiomyocytes of all α -actinin⁺ cardiomyocytes are quantified (**j**). No increase of proliferation is found upon FSTL1 treatment. (n=4) *: statistically different from control, P<0.05.

k-m) Sca1⁺ progenitor cells¹⁸ were starvation-synchronized for 48 hours and stimulated with FSTL1 or control growth medium for **72 hours** in presence of EdU. Clone 3 was obtained by clonal growth from the Lin-Sca1+SP fraction. Sca1 pool was obtained from lin-Sca1+ without clonal growth. **k)** EdU and DAPI staining of Sca1⁺ cells after 72 hours treatment. **l)** Percentage of EdU⁺ Sca1⁺ cells after 72 hours treatment. FSTL1 concentration: 0, 1, 10, 100 ng/ml. Abbreviations: SS, serum starvation; CGM, control growth medium. **m)** Number of Sca1⁺ cells after 72 hours FSTL1 treatment (n=5). No significant change is found upon FSTL1 treatment.

Extended Data Table 1. Raw echocardiography values (average \pm SEM) obtained at days 0 (baseline), 14, and 28 post treatment in a mouse model of permanent LAD ligation. The patch was implanted simultaneously with injury. *: statistically significant difference (P<0.05) in comparison with Sham. •: statistically significant difference (P<0.05) in comparison with MI-only. ■: statistically significant difference (P<0.05) in comparison with MI+Patch. ▲: statistically significant difference (P<0.05) in comparison with MI+Patch+CM.

Extended Data Table 2. Raw echocardiography values (average \pm SEM) in a long term (months 2 and 3) post treatment, in a mouse model of permanent LAD ligation. The patch was implanted simultaneously with injury. *: statistically significant difference (P<0.05) in comparison with Sham. •: statistically significant difference (P<0.05) in comparison with MI-only. ■: statistically significant

difference ($P < 0.05$) in comparison with MI+Patch. ▲: statistically significant difference ($P < 0.05$) in comparison with MI+Patch+CM.

Extended Data Table 3. Raw echocardiography values (average \pm SEM) of delayed grafting in a mouse model of ischemia/reperfusion. Data obtained at baseline (pre-injury, pre-grafting), weeks 1 (post injury, pre grafting) and weeks 3, 5 (post-injury, post-grafting). The patch was implanted 1 week after injury. *: statistically significant difference ($P < 0.05$) in comparison with Sham. ●: statistically significant difference ($P < 0.05$) in comparison with MI-only. ■: statistically significant difference ($P < 0.05$) in comparison with MI+Patch. ▲: statistically significant difference ($P < 0.05$) in comparison with MI+Patch+CM.









